REMARKS/ARGUMENTS

Claims 1, 3, 5-7 and 10-22 are active in this application. Claims 1, 6, 7 and 10 have been amended for clarity. Support for the amendment in Claim 1 is found in Claim 2 and the specification as originally filed. Support for Claims 11-22 is found in Claims, 1, 5, 6 and 7. The specification has been amended to include a cross-reference to the PCT priority application. No new matter is added. Favorable reconsideration is requested.

The rejection of Claims 1-3, 5, 7 and 10 under 35 U.S.C. § 112, first paragraph ("enablement") is respectfully traversed.

First, SEQ ID NO: 1 is not the sequence of the peptide which had been tested against rheumatoid arthritis specific autoantibodies but represents the results of an amino terminal sequencing of w64-78 antigen which permitted the present inventors to identify that the antigen was, in fact, a citrullinated simple α-fibrin. Support for this is shown on page 12-13 of the application.

As claimed herein the present invention provide a purified citrullinated polypeptide which reacts with rheumatoid arthritis specific autoantibodies and is selected from one of

citrullinated α-chain of a mammalian fibrin;

a citrullinated α-chain of a mammalian fibrinogen;

and a fragment of at least 5 consecutive amino acids of the α-chain of a mammalian fibrin which also comprises at least one citrulline residue.

The specification describes in detail how to purify citrullinated α- fibrin, obtain citrullinated recombinant fibrinogen, and how to test reactivity with rheumatoid arthritis specific autoantibodies. Specifically, Applicants draw the Examiner's attention to pages 3 and 6-10 for disclosure of the purification, pages 10-13 for the characterization of these polypeptides, pages 13-14 for the testing and reactivity with rheumatoid arthritis serum; and pages 15-17 for the deimination of fibrinogen.

Concerning Claim 1 c), obtaining a fragment of more than 5 amino acids of α- fibrin that react with rheumatoid arthritis specific autoantibodies does not require undue experimentation because one can simply cut fibrin into fragments, for example, using a protease. Equally feasible is to synthesize a peptide representing known of fibrin sequences which comprise at least one arginine residue in which the peptides are subsequently citrullinated or citrullinated during synthesis. Several examples of these known sequences are attached herewith as entries from the PubMed database. Further, based on these and other sequences it is known that human fibrin and fibrin from other vertebrates are sure of several regions of strong emology which also comprises arginine residues.

For example, in Example 2 of the present application it is shown that peptidyl arginine deiminase (PAD) citrullination of α- fibrinogen (which has a more complex structure than fibrin fragments and fibrin) enables it to react with RA-specific autoantibodies. For reference, see the text from pages 15-16 reproduced below:

> After deimination for 2 hours, the electrophoretic mobility by SDS-PAGE of the two α- and β-polypeptides became modified and that of the γ-polypeptide remained unchanged. Specifically, the protein corresponding to the α-chain then appeared in the form of a diffuse band of 82 to 95 kDa and was immunodetected by both the "311" antifibrinogen monoclonal antibody (figure 3B) and the antiserum directed against the α-chain of fibrinogen (results not shown).

Furthermore, partial citrullination of arginine residues resulting in a charge heterogeneity does not affect the reactivity to RA specific antibodies. As shown in Example 1 (page 11 lines 20-33 and Figure 2), the antigens w64-78 and 55-61 (citrullinated α-fibrin and β-fibrin extracted from synovial tissue) recognized by antifilaggrin autoantibodies (AFA) have heterogeneous pI, which reflects different level of citrullination:

> After staining with amido black, the presence of two major proteins, with an apparent molecular weight of 64-78 kD and

> 55-61 kD and pI of approximately 5.85 to approximately 8.45, is observed.
>
> These proteins are immunodetected with the AFA-positive rheumatoid sera but not with the AFA-negative rheumatoid sera.

It is also noted that the Examiner's reliance on the identification of T cell epitopes (page 4 of the Office Action referencing Schellekens) is irrelevant since the claimed peptides are not T cell epitopes with B cell epitopes which are recognized by the antibodies.

Finally, with respect to the ability of the citrullinated polypeptides of the present invention to be used as a pharmaceutical, Applicants direct the Examiner's attention to the specification on page 5 where the ability of the citrullinated polypeptides to neutralize the autoimmune response in RA-type diseases is described. Further, based on their identification and role in the autoimmune response it is reasonable that the polypeptides can be used in such a manner (se pages 1-2 of the present specification concerning the autoimmune response related to RA).

In view of the above, the present claims are enabled and as such withdrawal of this ground of rejection is requested.

The rejection of Claims 1-3, 5, 7 and 10 under 35 U.S.C. § 112, first paragraph ("written description") is respectfully traversed.

A relevant inquiry to written description is whether one of skill in the art would recognize that the Applicants' had possession of the claimed invention. Here, there is no question that Applicants' had possession of citrullinated polypeptides of the α chain of firbin, α chain of fibrinogen and fragments of the α-chain of fibrin that contain at least 5 consecutive amino acids and at least one citrulline residue. This is supported by the relevant disclosure in the specification, which is discussed in detail above.

To reiterate, the application clearly describes that the citrullination of fibrin and fibrinogen sequences provides antigens that react specifically with rheumatoid arthritis autoantibodies. Further, fibrin and fibrinogen alpha chain sequences are known. Accordingly, withdrawal of this ground of rejection is requested.

The rejection of Claims 1-3, 5 and 10 under 35 U.S.C. § 102(a) over Masson-Bessiare et al. is respectfully traversed.

This publication was published in December 1999. The present application is a 371 application of PCT/FR 00/01857 filed June 30, 2000 and claims priority to French Application 99/08470 filed July 1, 1999. In order to perfect priority to the French priority application, Applicants submit herewith a certified English translation thereof. Accordingly, Applicants request that the present application be given the benefit of the priority date and as such withdrawal of this ground of rejection is also requested.

The rejections of Claims 5 and 7 under 35 U.S.C. § 103(a) over Masson-Bessiare et al. are respectfully traversed. As noted, *supra*, the present application has claimed priority to French application filed July 1999. Upon finding the application deserves benefit to this priority application, Applicants request withdrawal of both grounds of rejection.

The rejection of Claims 1-3 and 10 or Claim 5 under 35 U.S.C. § 103(a) over U.S. Patent No. 5,821,068 (U.S. '068) in view of Schellekens et al. are respectfully traversed.

U.S. '068 describes antibodies that react with native (non-citrullinated) fibrin or fibrin fragments (see col. 2, lines 30-55). U.S. '068 does not describe or suggest that fibrin exists in a deiminated form.

Schellekens et al. describe the reactivity of APF/AKA autoantibodies with citrullinated synthetic peptides derived from filaggrin. However, Schellekens et al also does not describe citrullinated fibrin or fibrinogen sequences nor there involvement in RA. This is supported by Schellekens et al. who state on page 279, 2nd column, 3rd paragraph: "that the

antibodies reactive towards the citrullinated epitopes originate from a response against yet an unidentified, cross-reactive protein (or proteins)." Further, Schellekens et al indicate that "such a protein should contain deiminated arginine residues and some sequence resemblance to the peptides described here." Schellekens et al, however, provide nothing to the identification of the cross-reactive protein nor the role of citrullinated α-chain fibrin and/or fibrinogen in RA.

Thus, Schellekens et al combined with U.S. '068 would not have provided any suggestion that citrullinated fibrin is the cross-reactive protein with RA-specific autoantibodies. The Examiner's basis for the rejection is simply that one would have searched for the deiminated form of the α-chain polypeptide "because the modification and the identification of such peptides will not only enhance their diagnostic usefulness, but will also provide more precise information on the nature of the antigenic determinants responsible for the specific occurrence of APF/AKA antibodies in RA sera as taught by schellekens et al." (page 10 of the Official Action). However, this is, at best, an invitation to experiment and search for the determinants but provides no reasonable suggestion for the role of citrullinated fibrin is the cross-reactive protein with RA-specific autoantibodies. In fact, prior to the present invention, the existence of deiminated fibrin was not known and that the sequence in Schellekens et al. bears no resemblance to the sequence of fibrin.

Accordingly, withdrawal of this ground of rejection is requested.

Similarly, the rejections of Claims 5 or 7 under 35 U.S.C. § 103(a) over U.S. Patent No. 5,821,068 (U.S. '068) in view of Schellekens et al. further in view of U.S. patent no. 5,858,723 or U.S. 4,281,061 are respectfully traversed.

The deficiencies of U.S. '068 and Schellekens et al in describing the claimed citrullinated polypeptides is discussed in detail above.

U.S. '723 describes labeled polypeptide s for diagnosing seminoma. Examples of those labeled peptides are env and/or gag (see Example 4). However, U.S. '723 combined with U.S.'068 and Schellekens et al provides no suggestion for labeled citrullinated α-chain fibrin, fibrinogen or at least a 5 amino acid fragment of α-chain fibrin as claimed.

U.S. '061 describes immunoassay reagents provided in kits and lists several proteins or analyte that could be used (col. 7-8), one of which is fibrinogen (col. 8, line 58). However, U.S. '061 combined with U.S.'068 and Schellekens et al provides no suggestion for citrullinated α-chain fibrin, fibrinogen or at least a 5 amino acid fragment of α-chain fibrin as claimed.

In view of the above, withdrawal of both grounds of rejection is requested.

The rejection of Claims 1-3, 5, 7 and 10 under 35 U.S.C. § 101 is addressed by amendment.

The rejection of Claim 5 under 35 U.S.C. § 112, second paragraph is addressed by amendment.

Applicants also request that this application be passed to issuance. Early notification of such allowance is kindly requested.

Respectfully submitted,

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LOCUS NP_068657 644 aa linear PRI 21-DEC-2003
DEFINITION fibrinogen, alpha chain isoform alpha preproprotein [Homo sapiens].
ACCESSION NP_068657
VERSION NP_068657.1 GI:11761629
DBSOURCE REFSEQ: accession NM_021871.1
KEYWORDS .
SOURCE Homo sapiens (human)
ORGANISM Homo sapiens
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE 1 (residues 1 to 644)
AUTHORS Standeven,K.F., Grant,P.J., Carter,A.M., Scheiner,T., Weisel,J.W. and Ariens,R.A.
TITLE Functional analysis of the fibrinogen Aalpha Thr312Ala polymorphism: effects on fibrin structure and function
JOURNAL Circulation 107 (18), 2326-2330 (2003)
PUBMED 12707238
REMARK GeneRIF: Functional analysis of the fibrinogen Aalpha Thr312Ala polymorphism: effects on fibrin structure and function.
REFERENCE 2 (residues 1 to 644)
AUTHORS Attanasio,C., David,A. and Neerman-Arbez,M.
TITLE Outcome of donor splice site mutations accounting for congenital afibrinogenemia reflects order of intron removal in the fibrinogen alpha gene (FGA)
JOURNAL Blood 101 (5), 1851-1856 (2003)
PUBMED 12406899
REMARK GeneRIF: Analysis of the IVS3delGTAA mutation showed exon 3 skipping in 99% of transcripts & exons 2 & 3 skipping in 1% of transcripts. In FGA intron 3 was preferentially spliced first, followed by intron 2, intron 4, & intron 1.
REFERENCE 3 (residues 1 to 644)
AUTHORS Everse,S.J.
TITLE New insights into fibrin (ogen) structure and function
JOURNAL Vox Sang. 83 Suppl 1, 375-382 (2002)
PUBMED 12617173
REMARK GeneRIF: review of details of the structure, binding interactions, and function of each of the fibrinogen chains, FGA, FGB, FGG
REFERENCE 4 (residues 1 to 644)
AUTHORS Seydewitz,H.H., Gram,J., Bruhn,H.D. and Witt,I.
TITLE Fibrinogen variation: a heterozygote dysfibrinogenemia with Arg-->His substitution in position 16 of the Aalpha chain
JOURNAL Hamostaseologie 22 (2), 7-10 (2002)
PUBMED 12193970
REMARK GeneRIF: dysfibrinogenemia caused by variation: amino acid substition in position 16 Arg-His
REFERENCE 5 (residues 1 to 644)
AUTHORS Tsurupa,G., Tsonev,L. and Medved,L.
TITLE Structural organization of the fibrin(ogen) alpha C-domain
JOURNAL Biochemistry 41 (20), 6449-6459 (2002)
PUBMED 12009908
REMARK GeneRIF: Fibrinogen isoform alpha C-domain consists of a compact globular cooperative unit attached to the bulk of the molecule by an extended NH2-terminal connector region with a helical poly(L-proline) type II conformation.
REFERENCE 6 (residues 1 to 644)
AUTHORS Mathonnet,F., Peltier,J.Y., Detruit,H., de Raucourt,E., Alvarez,J.C., Mazmanian,G.M. and de Mazancourt,P.
TITLE Fibrinogen Saint-Germain I: a case of the heterozygous Aalpha GLY 12 --> VAL fibrinogen variant
JOURNAL Blood Coagul. Fibrinolysis 13 (2), 149-153 (2002)
PUBMED 11914657
REMARK GeneRIF: Although the FGA mutation is the same in fibrinogen Rouen and fibrinogen Saint-Germain I, the latter shows a different thrombin-induced fibrinopeptide release pattern and a mild factor V deficiency.
REFERENCE 7 (residues 1 to 644)
AUTHORS Homer,V.M., Brennan,S.O. and George,P.M.

TITLE Four novel polymorphisms in the fibrinogen Aalpha gene
JOURNAL Thromb. Haemost. 87 (2), 354-355 (2002)
PUBMED 11858505
REMARK GeneRIF: Four novel polymorphisms in the fibrinogen Aalpha gene are described: 2 SNPs at -3 and -1051 and a dinucleotide repeat at -946 and a TaqI polymorphism.
FERENCE 8 (residues 1 to 644)
AUTHORS Margaglione,M., Vecchione,G., Santacroce,R., D'Angelo,F., Casetta,B., Papa,M.L., Grandone,E. and Di Minno,G.
TITLE A frameshift mutation in the human fibrinogen Aalpha-chain gene (Aalpha(499)Ala frameshift stop) leading to dysfibrinogen San Giovanni Rotondo
JOURNAL Thromb. Haemost. 86 (6), 1483-1488 (2001)
PUBMED 11776317
REMARK GeneRIF: The new dysfunctional fibrinogen, San Giovanni Rotondo variant, a heterozygous single-base deletion at Ala499 in the Aalpha-chain gene, predicts AA changes encoded by the rest of exon V and a premature stop at 518 (Aalpha[499]Ala frameshift stop).
FERENCE 9 (residues 1 to 644)
AUTHORS Remijn,J.A., van Wijk,R., de Groot,P.G. and van Solinge,W.W.
TITLE Nature of the fibrinogen Aalpha gene TaqI polymorphism
JOURNAL Thromb. Haemost. 86 (3), 935-936 (2001)
PUBMED 11583334
REMARK GeneRIF: The TaqI polymorphism is due to a 28bp duplication at 6587-6614.
FERENCE 10 (residues 1 to 644)
AUTHORS Liu,Y., Saha,N., Heng,C.K., Hong,S. and Low,P.S.
TITLE Fibrinogen genotypes (alpha and beta) are associated with plasma fibrinogen levels in Chinese
JOURNAL J. Med. Genet. 38 (9), E31 (2001)
PUBMED 11546832
REMARK GeneRIF: genotypes are associated with plasma fibrinogen levels in Chinese
FERENCE 11 (residues 1 to 644)
AUTHORS Herrick,S., Blanc-Brude,O., Gray,A. and Laurent,G.
TITLE Fibrinogen
JOURNAL Int. J. Biochem. Cell Biol. 31 (7), 741-746 (1999)
PUBMED 10467729
FERENCE 12 (residues 1 to 644)
AUTHORS Uemichi,T., Liepnieks,J.J., Yamada,T., Gertz,M.A., Bang,N. and Benson,M.D.
TITLE A frame shift mutation in the fibrinogen A alpha chain gene in a kindred with renal amyloidosis
JOURNAL Blood 87 (10), 4197-4203 (1996)
PUBMED 8639778
FERENCE 13 (residues 1 to 644)
AUTHORS Baumann,R.E. and Henschen,A.H.
TITLE Human fibrinogen polymorphic site analysis by restriction endonuclease digestion and allele-specific polymerase chain reaction amplification: identification of polymorphisms at positions A alpha 312 and B beta 448
JOURNAL Blood 82 (7), 2117-2124 (1993)
PUBMED 8400261
FERENCE 14 (residues 1 to 644)
AUTHORS Benson,M.D., Liepnieks,J., Uemichi,T., Wheeler,G. and Correa,R.
TITLE Hereditary renal amyloidosis associated with a mutant fibrinogen alpha-chain
JOURNAL Nat. Genet. 3 (3), 252-255 (1993)
PUBMED 8097946
FERENCE 15 (residues 1 to 644)
AUTHORS Fu,Y., Weissbach,L., Plant,P.W., Oddoux,C., Cao,Y., Liang,T.J., Roy,S.N., Redman,C.M. and Grieninger,G.
TITLE Carboxy-terminal-extended variant of the human fibrinogen alpha subunit: a novel exon conferring marked homology to beta and gamma subunits
JOURNAL Biochemistry 31 (48), 11968-11972 (1992)
PUBMED 1457396

FERENCE 16 (residues 1 to 644)
AUTHORS Chung,D.W., Harris,J.E. and Davie,E.W.
TITLE Nucleotide sequences of the three genes coding for human fibrinogen
JOURNAL (in) Liu,C.Y. and Chien,S. (Eds.);
FIBRINOGEN, THROMBOSIS, COAGULATION AND FIBRINOLYSIS: 39-48;
Plenum Press, New York (1991)
FERENCE 17 (residues 1 to 644)
AUTHORS Weissbach,L. and Grieninger,G.
TITLE Bipartite mRNA for chicken alpha-fibrinogen potentially encodes an amino acid sequence homologous to beta- and gamma-fibrinogens
JOURNAL Proc. Natl. Acad. Sci. U.S.A. 87 (13), 5198-5202 (1990)
PUBMED 2367530
FERENCE 18 (residues 1 to 644)
AUTHORS Humphries,S.E., Imam,A.M., Robbins,T.P., Cook,M., Carritt,B., Ingle,C. and Williamson,R.
TITLE The identification of a DNA polymorphism of the alpha fibrinogen gene, and the regional assignment of the human fibrinogen genes to 4q26-qter
JOURNAL Hum. Genet. 68 (2), 148-153 (1984)
PUBMED 6500566
FERENCE 19 (residues 1 to 644)
AUTHORS Doolittle,R.F.
TITLE Fibrinogen and fibrin
JOURNAL Annu. Rev. Biochem. 53, 195-229 (1984)
PUBMED 6383194
FERENCE 20 (residues 1 to 644)
AUTHORS Imam,A.M., Eaton,M.A., Williamson,R. and Humphries,S.
TITLE Isolation and characterisation of cDNA clones for the A alpha- and gamma-chains of human fibrinogen
JOURNAL Nucleic Acids Res. 11 (21), 7427-7434 (1983)
PUBMED 6689067
FERENCE 21 (residues 1 to 644)
AUTHORS Kant,J.A., Lord,S.T. and Crabtree,G.R.
TITLE Partial mRNA sequences for human A alpha, B beta, and gamma fibrinogen chains: evolutionary and functional implications
JOURNAL Proc. Natl. Acad. Sci. U.S.A. 80 (13), 3953-3957 (1983)
PUBMED 6575389
FERENCE 22 (residues 1 to 644)
AUTHORS Chung,D.W., Rixon,M.W., Que,B.G. and Davie,E.W.
TITLE Cloning of fibrinogen genes and their cDNA
JOURNAL Ann. N. Y. Acad. Sci. 408, 449-456 (1983)
PUBMED 6575700
FERENCE 23 (residues 1 to 644)
AUTHORS Rixon,M.W., Chan,W.Y., Davie,E.W. and Chung,D.W.
TITLE Characterization of a complementary deoxyribonucleic acid coding for the alpha chain of human fibrinogen
JOURNAL Biochemistry 22 (13), 3237-3244 (1983)
PUBMED 6688355
MMENT REVIEWED REFSEQ: This record has been curated by NCBI staff. The reference sequence was derived from M64982.1.

Summary: The protein encoded by this gene is the alpha component of fibrinogen, a blood-borne glycoprotein comprised of three pairs of nonidentical polypeptide chains. Following vascular injury, fibrinogen is cleaved by thrombin to form fibrin which is the most abundant component of blood clots. In addition, various cleavage products of fibrinogen and fibrin regulate cell adhesion and spreading, display vasoconstrictor and chemotactic activities, and are mitogens for several cell types. Mutations in this gene lead to several disorders, including dysfibrinogenemia, hypofibrinogenemia, afibrinogenemia and renal amyloidosis. Alternative splicing results in two isoforms which vary in the carboxy-terminus.

Transcript Variant: This variant (alpha) lacks exon 6, resulting in the shorter isoform (alpha) with a different carboxy-terminus compared to isoform alpha-E.
ATURES Location/Qualifiers

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                  /db_xref="taxon:9606"
                  /chromosome="4"
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                  preproprotein"
  sig_peptide     1..19
  variation       6
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  proprotein      20..644
  mat_peptide     20..35
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                  /product="fibrinogen, alpha chain, isoform alpha"
                  /note="thrombin cleavage product"
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  variation       392
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                  /replace="S"
                  /db_xref="dbSNP:10921"
  variation       446
                  /replace="K"
                  /replace="E"
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                  /note="isoform alpha preproprotein is encoded by
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                  [evidence E] [pmid 10467729];
                  go_function: fibrinogen [goid 0008001] [evidence NR];
                  go_function: cell adhesion molecule activity [goid
                  0005194] [evidence NR];
                  go_process: positive regulation of cell proliferation
                  [goid 0008284] [evidence NR];
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                  [evidence NR];
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    121 nnrdntynrv sedlrsriev lkrkviekvq hiqllqknvr aqlvdmkrle vdidikirsc
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    241 lqkvppewka ltdmpqmrme lerpggneit rggstsygtg setesprnps sagswnsgss
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    421 spgtrreyht eklvtskgdk elrtgkekvt sgsttttrrs csktvtktvi gpdghkevtk
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541 vsetesrgse sgiftntkes sshhpgiaef psrgksssys kqftsstsyn rgdstfesks
601 ykmadeagse adhegthstk rghaksrpvr gihtsplgkp slsp

```
CUS        D44234                    866 aa            linear   PRI 19-JAN-2001
FINITION   fibrinogen alpha chain precursor, extended splice form - human.
CESSION    D44234
RSION      D44234  GI:2135107
SOURCE     pir: locus D44234;

           summary: #length 866 #molecular-weight 94972 #checksum 6068
           ;
           genetic: #gene GDB:FGA ##cross-references GDB:119129; OMIM:134820
           #map_position 4q28-4q28 #introns 18/3; 60/3; 122/1; 171/2 #note the
           list of introns is incomplete
           ;
           includes: fibrinopeptide A
           ;
           superfamily: human extended splice form fibrinogen alpha chain;
           fibrinogen beta/gamma homology; fibrinogen disulfide ring homology
           ;
           PIR dates: 10-Jun-1993 #sequence_revision 06-Sep-1996 #text_change
           19-Jan-2001
           .
YWORDS     alternative splicing; blood coagulation; glycoprotein; liver;
           phosphoprotein; plasma.
URCE       Homo sapiens (human)
ORGANISM   Homo sapiens
           Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
           Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
FERENCE    1  (residues 1 to 866)
AUTHORS    Fu,Y., Weissbach,L., Plant,P.W., Oddoux,C., Cao,Y., Liang,T.J.,
           Roy,S.N., Redman,C.M. and Grieninger,G.
TITLE      Carboxy-terminal-extended variant of the human fibrinogen alpha
           subunit: a novel exon conferring marked homology to beta and gamma
           subunits
JOURNAL    Biochemistry 31 (48), 11968-11972 (1992)
MEDLINE    93090725
 PUBMED    1457396
MMENT      The alpha chain binds by 2-4 cross-links to the amino end of
           fibronectin.
           The conversion of fibrinogen to fibrin is triggered by thrombin,
           which cleaves fibrinopeptides A and B from alpha and beta chains,
           respectively, and thus exposes the amino-terminal polymerization
           sites responsible for the formation of the soft clot.
           The soft clot is converted into the hard clot by factor XIIIa
           (fibrin-stabilizing factor, FSF), which catalyzes the
           epsilon-(gamma-glutamyl)lysine cross-linking between gamma chains
           (stronger) and between alpha chains (weaker) of different monomers.
           All fibrinogen chains are synthesized in the liver.
           See PIR:FGHUA for the major splice form. It is not known whether
           this form is glycosylated.
           The fibrinogen molecule is a hexamer containing two sets of three
           nonidentical chains (alpha, beta, and gamma), linked to each other
           by disulfide bonds. The amino ends of all chains are contained in
           the core. Two three-chain coiled coils emerge from this core and
           connect it to nodes containing the distal domains. The long
           carboxyl ends of the alpha chains extend peripherally from the
           distal domain nodes.
ATURES                 Location/Qualifiers
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                       /db_xref="taxon:9606"
   Protein             1..866
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   Region              20..863
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Site            22
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                /note="phosphate (Ser) (covalent)"
Site            35..36
                /site_type="cleavage"
                /note="Arg-Gly (thrombin)"
Region          36..863
                /region_name="product"
                /note="fibrin alpha chain, extended splice form"
Bond            bond(47)
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                /note="interchain (to alpha-47)"
Bond            bond(55)
                /bond_type="disulfide"
                /note="interchain (to beta-95)"
Region          57..185
                /region_name="domain"
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Bond            bond(64)
                /bond_type="disulfide"
                /note="interchain (to gamma-49)"
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    181 rgscsralar evdlkdyedq qkqleqviak dllpsrdrqh lplikmkpvp dlvpgnfksq
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:YWORDS     .
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:FERENCE    1  (sites)
 AUTHORS    Weissbach,L. and Grieninger,G.
 TITLE      Bipartite mRNA for chicken alpha-fibrinogen potentially encodes an
            amino acid sequence homologous to beta- and gamma-fibrinogens
 JOURNAL    Proc. Natl. Acad. Sci. U.S.A. 87 (13), 5198-5202 (1990)
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  PUBMED    2367530
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    661 vycdqetslg gwlliqqrmd gslnfnrtwq dykrgfgsln degegefwlg ndylhlltqr
    721 gsvlrveled wagneayaey hfrvgseaeg yalqvssyeg tagdaliegs veegaeytsh
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:RSION AAC97143.1 GI:4033511
)SOURCE locus HUMFBRABI accession M58569.1
:YWORDS .
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ORGANISM Homo sapiens
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
:FERENCE 1 (sites)
AUTHORS Weissbach,L. and Grieninger,G.
TITLE Bipartite mRNA for chicken alpha-fibrinogen potentially encodes an
amino acid sequence homologous to beta- and gamma-fibrinogens
JOURNAL Proc. Natl. Acad. Sci. U.S.A. 87 (13), 5198-5202 (1990)
MEDLINE 90311369
PUBMED 2367530
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Method: conceptual translation.
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CDS 1..644
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FINITION  common fibrinogen alpha chain.
CCESSION  AAA17055
RSION     AAA17055.1  GI:458554
SOURCE    locus HUMFIBRA accession M64982.1
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URCE      Homo sapiens (human)
ORGANISM  Homo sapiens
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FERENCE   1  (sites)
AUTHORS   Chung,D.W. and Grieninger,G.
TITLE     Fibrinogen DNA and protein sequences
JOURNAL   (in) Ebert,R.F. (Ed.);
          INDEX OF VARIANT HUMAN FIBRINOGENS: 13-24;
          CRC Press, Boca Raton (1994)
FERENCE   2  (sites)
AUTHORS   Baumann,R.E. and Henschen,A.H.
TITLE     Human fibrinogen polymorphic site analysis by restriction
          endonuclease digestion and allele-specific polymerase chain
          reaction amplification: identification of polymorphisms at
          positions A alpha 312 and B beta 448
JOURNAL   Blood 82 (7), 2117-2124 (1993)
MEDLINE   94003263
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MMENT     On Mar 8, 1994 this sequence version replaced gi:182596.
          Revised based on bipartite transcript -- M58569 -- to include
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          extended variant, alpha-E, which is identical to the alpha chain
          through Arg611 but has an additional 236 amino acids that are
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          Relationship of both alpha chain protein sequences to the gene
          sequence is illustrated in citation [3].
          Method: conceptual translation.
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:YWORDS     .
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 ORGANISM   Homo sapiens
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:FERENCE    1  (residues 1 to 644)
 AUTHORS    Rixon,M.W., Chan,W.Y., Davie,E.W. and Chung,D.W.
 TITLE      Characterization of a complementary deoxyribonucleic acid coding
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 JOURNAL    Biochemistry 22 (13), 3237-3244 (1983)
 MEDLINE    83283432
  PUBMED    6688355
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```

CERTIFICATE OF VERIFICATION

I, Willy BARBOT

of Cabinet ORES – 36 rue de Saint-Pétersbourg , 75008 PARIS (FR)

state that the attached document is a true and complete translation to the best of my knowledge of French Patent Application n° 99 08470 of July 1st, 1999.

Dated this 26th day of January 2004

Signature of Translator : _____

FIBRIN CITRULLINE DERIVATIVES AND THEIR USE FOR DIAGNOSING OR TREATING RHEUMATOID ARTHRITIS

The present invention relates to citrullinated derivatives of fibrin and to their uses in diagnosing and treating rheumatoid arthritis.

Rheumatoid arthritis (hereinafter abbreviated to "RA") is the most common of the forms of chronic inflammatory rheumatism. It is an autoimmune disease; the serum of affected patients contains autoantibodies, some of which are specific and may constitute a marker for this disease, allowing it to be diagnosed even at early stages.

Prior studies by the team of the inventors have shown that these antibodies recognize different molecular forms of the (pro)filaggrin family (for review, cf. for example SERRE and VINCENT, In: Autoantibodies, PETER and SHOENFIELD Eds, Elsevier Science Publishers, 271-276, 1996). These antibodies have, for this reason, been named: "antifilaggrin autoantibodies (AFAs)". Application EP 0 511 116 describes the purification and characterization of antigens of the filaggrin family, recognized by these antibodies, and their use for diagnosing rheumatoid arthritis.

The inventors have shown that the epitopes recognized by the AFAs are carried by regions of the filaggrin molecule, in which at least some of the arginines are deiminated and thus transformed into citrulline; citrullinated peptides specifically recognized by AFAs have thus been obtained from the main immunoreactive regions of filaggrin. These peptides, and their use for diagnosing RA, are the subject of Application PCT/FR97/01541 and of Application PCT/FR98/02899 in the name of BIOMERIEUX. The inventors' observations concerning the role of citrulline residues in the reactivity of filaggrin with RA-specific autoantibodies

have subsequently been confirmed by other researchers [SCHELLEKENS et al., Arthritis Rheum., 40, no. 9 supplement, p. S276, summary 1471 (1997); VISSER et al., Arthritis Rheum., 40, no. 9 supplement, p. S289, summary 1551 (1997)].

The inventors have also shown that AFAs represent a considerable proportion of the interstitial immunoglobulins of synovial rheumatoid tissues and that they are synthesized locally by specific plasmocytes present in these tissues, which confirms the hypothesis that they are involved in the autoimmune response associated with RA. The use of filaggrin, or of citrullinated peptides derived therefrom, to neutralize this autoimmune response is the subject of Application PCT/FR98/02900 in the name of UNIVERSITÉ PAUL SABATIER [Paul Sabatier University](TOULOUSE III).

However, the involvement of filaggrin as an immunogen or as a target antigen in the autoimmune response associated with RA has never been noted. The true antigen involved in this response remains to be identified.

The inventors have now succeeded in characterizing this antigen and have thus shown that it is composed of citrullinated derivatives of the α- and/or β-chains of fibrin.

A subject of the present invention is a citrullinated polypeptide derived from all or part of the sequence of the α-chain or of the β-chain of a vertebrate fibrin, by substitution of at least one arginine residue with a citrulline residue.

Preferably, a polypeptide in accordance with the invention comprises at least 5 consecutive amino acids and advantageously at least 10 consecutive amino acids,

including at least one citrulline, of the sequence of the α-chain or of the β-chain of a mammalian fibrin. Advantageously, said vertebrate fibrin is a mammalian fibrin, preferably a human fibrin.

Citrullinated polypeptides in accordance with the invention may, for example, be obtained from natural, recombinant or synthetic fibrin or fibrinogen, or from fragments thereof, comprising at least one arginine residue, by the action of peptidyl arginine deiminase (PAD); they may also be obtained by peptide synthesis, directly incorporating one or more citrulline residues into the synthesized peptide.

Citrullinated polypeptides in accordance with the invention may also be pseudopeptides having the same three-dimensional structure, and therefore the same immunological reactivity, as the citrullinated polypeptides derived from the α- or β-chains of fibrin, or from fragments thereof, mentioned above. They may, for example, be pseudopeptides of the *retro* type, in which L-amino acids are linked together according to a reverse sequence of that of the peptide to be reproduced, or pseudopeptides of the *retro-inverso* type, consisting of D-series amino acids (instead of the L-series amino acids of natural peptides) linked together according to a reverse sequence of that of the peptide to be reproduced, or alternatively pseudopeptides containing a CH_2-NH bond in place of a CO-NH peptide bond. Pseudopeptides of these various types are, for example, described by BENKIRANE et al. [J. Biol. Chem., 270, p. 11921-11926, (1995); J. Biol. Chem., 271, p. 33218-33224, (1996)]; BRIAND et al. [(J. Biol. Chem., 270, p. 20686-20691, (1995); GUICHARD et al. [J. Biol. Chem., 270, p. 26057-26059, (1995)].

A subject of the present invention is also the use of the polypeptides in accordance with the invention, as defined above, for diagnosing RA, *in vitro*.

The present invention in particular encompasses antigenic compositions for diagnosing the presence of RA-specific autoantibodies in a biological sample, which compositions are characterized in that they contain at least one polypeptide in accordance with the invention, optionally labeled with and/or conjugated to a carrier molecule.

A subject of the present invention is also a method for detecting RA-specific autoantibodies of the G class in a biological sample, which method is characterized in that it comprises:

- bringing said biological sample into contact with at least one polypeptide in accordance with the invention, as defined above, under conditions which allow the formation of an antigen/antibody complex with the RA-specific autoantibodies possibly present;

- detecting, by any suitable means, the antigen/antibody complex possibly formed.

This detection method may be carried out using a kit comprising at least one antigen according to the invention, and also buffers and reagents suitable for constituting a reaction medium which allows the formation of an antigen/antibody complex, and/or means for detecting said antigen/antibody complex.

Said kit may also comprise, where appropriate, reference samples, such as one or more negative serum (sera) and one or more positive serum (sera).

A subject of the present invention is also the use of citrullinated polypeptides in accordance with the invention, for producing a medicinal product, and especially a medicinal product intended to neutralize the autoimmune response associated with RA, and in particular to inhibit the attachment of the humoral or cellular effectors of this autoimmune response, to the citrullinated derivatives of α- or β-chains of fibrin which are present in rheumatoid tissues.

This *in vivo* neutralization of the autoimmune response may contribute to treating RA or other diseases which are thought to involve lesions induced by an autoimmune response directed against epitopes exhibiting cross-reactions with the citrullinated derivatives of α- or β-chains of fibrin.

Advantageously, for *in vivo* administration, polypeptides modified so as to prolong their lifetime in the organism, in particular by increasing their resistance to proteases, will be chosen; they may in particular be pseudopeptides, such as those mentioned above.

The present invention also encompasses pharmaceutical compositions, in particular for treating rheumatoid arthritis, characterized in that they contain, as active principle, at least one polypeptide in accordance with the invention.

Pharmaceutical compositions in accordance with the invention may be administered by any suitable means known per se. They may, for example, be administered systemically, orally, parenterally, or by subcutaneous, intravenous or intramuscular injection; they may also be administered locally, for example by intra-articular injections or by microinjections, under arthroscopy, into the inflammatory synovial tissue.

The present invention will be more clearly understood using the additional description which follows, which refers to the identification of deiminated forms of the α-chain or β-chain of human fibrin in rheumatoid tissues, and to the use of deiminated fibrinogen for detecting the presence of AFAs in serum samples.

EXAMPLE 1: PURIFICATION AND CHARACTERIZATION OF ANTIGENIC PROTEINS RECOGNIZED BY AFAs IN RHEUMATOID SYNOVIAL TISSUES

1) Analysis of rheumatoid synovial tissues

Materials and methods:

The synovial tissue samples used for the protein extractions were taken from patients suffering from rheumatoid arthritis, during a synovectomy or an arthroplasty of the wrist or knee, and all correspond to tissue fragments which are the seat of conventional histological rheumatoid synovitis lesions. They are conserved by freezing in isopentane cooled with liquid nitrogen.

Synovial tissue fragments originating from four patients were extracted sequentially, in a low ionic strength buffer, a urea buffer and in a urea/DTT buffer, successively.

Preparation of synovial extracts

The extraction was carried out using an Ultra-Turrax homogenizer (T25 basic, IKA Labortechnik, Staufen, Germany) with a volume of 6 ml of buffer per gram of tissue.

The following buffers were used at a temperature of 0°C: 40 mM Tris-HCl, pH 7.4, containing 150 mM of NaCl [low

ionic strength buffer]; 40 mM Tris-HCl, pH 7.4, containing 8M urea deionized on an ion exchange resin (AG 501-X8, Biorad, Hercules, CA) [urea buffer]; 40 mM Tris-HCl, pH 7.4, containing 8M deionized urea and 50 mM dithiothreitol (DTT), (Sigma) [urea/DTT buffer]. All the buffers were supplemented with 20 mM EDTA, 0.02% sodium azide, 2 µg/ml aprotinin, 10 mM N-ethylmaleimide and 1 mM phenylmethylsulfonyl fluoride (Sigma, Saint Louis, MI). After each extraction, the homogenates were centrifuged for 20 minutes at 15,000 g, at the temperature of 4°C. The urea buffer and urea/DTT buffer extracts were dialyzed against water before being analyzed by electrophoresis and by immunotransfer.

Electrophoresis and immunodetection

The synovial proteins of the various extracts were separated by electrophoresis on a 10% polyacrylamide gel in denaturing SDS buffer (SDS-PAGE), and were then electrotransferred onto reinforced nitrocellulose membranes (Hybond-™C extra, Amersham, Little Chalfont, UK).

The membranes were immunodetected with the following antibody preparations; AFA-positive or AFA-negative rheumatoid human sera; non-rheumatoid control human sera derived from patients suffering from other forms of inflammatory rheumatism or from healthy individuals (1/100); purified fractions of AFAs (10 µg/ml); mouse monoclonal antibody directed against human fibrin and fibrinogen (5 µg/ml); two sheep antisera directed, respectively, against recombinant α- and γ-chains of human fibrinogen (1/1000) (Cambio, Cambridge, UK); a rabbit antiserum directed against the recombinant β-chain of human fibrinogen (1/200000) (Cambio).

The human sera used are derived from 95 patients suffering from rheumatoid arthritis (RA), perfectly characterized from a clinical and biological point of view according to the criteria of the American College of Rheumatology, from 24 patients suffering from non-rheumatoid inflammatory rheumatism or from non-inflammatory pathological conditions (control sera) and from 10 healthy individuals. The semi-quantitative titration of the antifilaggrin antibodies (AFAs) in the sera was carried out by indirect imunofluorescence on cryosections of rat esophageal epithelium and by immunotransfer on epidermal extracts enriched in filaggrin acid variant, according to previously published protocols [VINCENT et al., Ann. Rheum. Dis., 48, 712-722 (1989); VINCENT et al., J. Rheumatol., 25, 838-846 (1998)]. The "AFA-positive" sera are those which exhibit AFAs at significant titers after detection using both methods, and the "AFA-negative" sera are those which do not exhibit detectable AFAs by either of the two methods.

The AFAs were purified by affinity chromatography on the epidermal filaggrin acid variant, according to the protocol described by GIRBAL-NEUHAUSER et al. (J. Immunol., 162, 585-594 (1999), using 45 rheumatoid sera having a high AFA titer. The purified antibody fractions were pooled.

Peroxidase-conjugated secondary molecular probes were used for detecting all the primary antibodies: protein A (Sigma), sheep antibodies directed against mouse IgGs (Biosys, Compiègne, France), goat Fab fragments directed against rabbit IgGs (Biosys) and rabbit F(ab')2 fragments directed against sheet IgGs (Southern Biotech. Inc), for detecting, respectively, human, murine, rabbit and sheep IgGs. The peroxidase activity was visualized using the ECL™ detection system

(Amersham International, Aylesbury, UK), according to the protocol provided by the manufacturer.

Results

Specific reactivity with the purified AFAs and the AFA-positive rheumatoid sera was observed only in the extract produced in urea/DTT buffer.

The results are illustrated by figure 1:

Legend to figure 1:
- AFAp = purified AFAs;
- RA sera = rheumatoid sera:
  * AFA+ = AFA-positive;
  * AFA- = AFA-negative;
- control sera = sera derived from patients suffering from forms of inflammatory rheumatism other than RA, or from healthy donors.

These results show that the specific reactivity with the purified AFAs and the AFA-positive rheumatoid sera relates to two protein bands of apparent molecular weight of approximately 64 kD to approximately 78 kD (w64-78) and of approximately 55 kD to approximately 61 kD (w55-61), respectively. These protein bands were not detected by the AFA-negative sera, regardless of whether they originate from patients suffering from RA or from other forms of inflammatory rheumatism, or are derived from healthy donors.

The presence of these proteins specifically recognized by the purified AFAs and the AFA-positive rheumatoid sera was observed in the urea/DTT extracts of synovial tissues derived from the 4 rheumatoid patients studied.

In total, 48 AFA-positive rheumatoid sera were tested by immunotransfer on at least one synovial urea/DTT

extract. Among the sera, 40 recognized w64-78, 39 recognized w55-61, 37 recognized both w64-78 and w55-61, 3 recognized only w64-78 and 2 recognised only w55-61.

Thirteen AFA-negative rheumatoid sera were tested by immunotransfer on at least one urea/DTT extract of synovial tissue; none of these sera recognized either w64-78 or w55-61.

Ten sera derived from healthy donors and 5 sera derived from patients suffering from other forms of inflammatory rheumatism were also tested by immunotransfer on at least one synovial urea/DTT extract; none of these sera recognized either w64-78 or w55-61.

2) Characterization of the w64-78 and w55-61 antigenic proteins

The proteins of the urea/DTT buffer extract of the synovial tissue of one of the patients suffering from RA were precipitated with 4 volumes of glacial acetone and then redissolved in the urea/DTT buffer at a concentration 15 times higher than their initial concentration.

The proteins of the concentrated extract were separated by two-dimensional electrophoresis, by isoelectrofocussing followed by SDS-PAGE.

A two-dimensional electrophoretic separation was carried out in the PhastSystem™ (Pharmacia). The first electrophoretic separation was performed on PhastGel™ isoelectrofocussing (IEF) gels which, beforehand, had been washed, dried and rehydrated in a deionized buffer containing 8 M urea, 0.5% Nonidet P-40 and ampholytes creating a pH gradient of 3 to 10 (Pharmacia). The

second dimension was performed by SDS-PAGE on 7.5% polyacrylamide gels.

The proteins were then electrotransferred onto polyvinyl difluoride (PVDF) membranes (ProBlott™ membranes, Applied Biosystems, Foster City, CA), in 50 mM Tris and 50 mM of boric acid. The membranes were finally stained with an aqueous solution of amido black at 0.1%, of acetic acid at 1% and of methanol at 45%, or immunodetected with rheumatoid sera according to the protocol described in 1) above.

Figure 2 illustrates the profiles obtained after electrotransfer onto a PVDF membrane and:

a) staining with amido black; or
b) immunodetection with an AFA-positive rheumatoid serum; or
c) immunodetection with an AFA-negative rheumatoid serum.

Legend to figure 2:
- Amido Black = staining with amido black;
- AFA+ = immunodetection with an AFA-positive rheumatoid serum;
- AFA- = immunodetection with an AFA-negative rheumatoid serum.

After staining with amido black, the presence of two major proteins, with an apparent molecular weight of 64-78 kD and 55-61 kD and pI of approximately 5.85 to approximately 8.45, is observed.

These proteins are immunodetected with the AFA-positive rheumatoid sera but not with the AFA-negative rheumatoid sera.

Using identical transfers onto a PVDF membrane after two-dimensional electrophoresis, membrane fragments corresponding to the center of each immunoreactive zone were excised and then subjected to amino-terminal sequencing in an Applied Biosystems sequencer (494A or 473A), according to the method recommended by the manufacturer.

The sequence gly-pro-arg-val-val-glu-arg-his-gln-ser-ala was obtained from the membrane fragment corresponding to the w64-78 antigen. This sequence is strictly identical to the sequence 36-46 of the product of the human fibrinogen α-chain precursor gene. When membrane fragments corresponding to the right or left ends of the w64-78 immunoreactive zone were excised and then each subjected to three cycles of amino-terminal sequencing, gly-pro-arg sequences were found each time, indicating that the entire p64-78 immunoreactive zone has the same amino-terminal end.

The sequence gly-his-arg-pro-leu-asp-lys-lys-arg was obtained from the membrane fragment corresponding to the center of the immunoreactive zone corresponding to the w55-61 antigen. This sequence is strictly identical to the sequence 45-54 of the product of the human fibrinogen β-chain precursor gene. When a membrane fragment corresponding to the left end of the w55-61 immunoreactive zone was excised and then subjected to two cycles of amino-terminal sequencing, the gly-his sequence was found. When a membrane fragment corresponding to the right end of the w55-61 immunoreactive zone was excised and then subjected to six cycles of amino-terminal sequencing, the gly-his-arg-pro-leu-asp sequence and the gly-pro-arg-val-val-glu sequence were found. This indicates that the entire w55-61 immunoreactive zone has the same amino-terminal end and that it partially co-migrates with the w64-78 antigen.

The amino-terminal ends of the w64-78 and w55-61 antigenic proteins correspond, respectively, to the amino-terminal ends of the α- and β-chains of human fibrinogen after respective cleavage, by thrombin, of fibrinopeptides A and B. The amino-terminal ends of the w64-78 and w55-61 antigenic proteins are therefore identical, respectively, to that of the α-chain and to that of the β-chain of human fibrin.

The apparent molecular weights of the w64-78 and w55-61 antigens are compatible with the respective theoretical molecular weight values for the α-chain and for the β-chain of human fibrin.

The identity of the w64-78 antigen and of the α-chain of fibrin, on the one hand, and that of the w55-61 antigen and of the β-chain of fibrin, on the other hand, were confirmed by analyzing the reactivity of antifibrin(ogen) antibodies with respect to these antigens. By immunotransfer, using an extract of synovial tissue prepared in urea/DTT, the "311" mouse monoclonal antibody, which recognizes the three chains α, β and weakly, γ of human fibrinogen and fibrin, is mainly reactive with respect to the w64-78 and w55-61 antigens. Similarly, two antisera, one from sheep and the other from rabbit, directed, respectively, against recombinant α- and β-chains of fibrinogen, recognized mainly a protein which co-migrates with the w64-78 antigen and a protein which co-migrates with the w55-61 antigen, respectively.

EXAMPLE 2: REACTIVITY OF RHEUMATOID SERA AND OF PURIFIED AFAs WITH DEIMINATED FIBRINOGEN *IN VITRO*

The reactivity with respect to deiminated and nondeiminated fibrinogen was studied by immunotransfer. The following were used: the purified AFA fractions, 37

AFA-positive rheumatoid sera of decreasing titer, 10 AFA-negative rheumatoid sera and 19 AFA-negative sera derived from patients suffering from forms of inflammatory or non-inflammatory rheumatism (AFA titers determined by immunotransfer on epidermal extracts enriched in filaggrin acid variant).

The results are illustrated by Figure 3A in the case of nondeiminated fibrinogen and by Figure 3B in the case of deiminated fibrinogen.

Legend to Figure 3:

Figure 3A: non deiminated purified human fibrinogen;

- 311 = antifibrinogen monoclonal antibody 311;
- control sera = sera derived from patients suffering from forms of inflammatory rheumatism other than RA, or from healthy donors;
- RA sera = rheumatoid sera;
    * AFA+ = AFA-positive;
    * AFA- = AFA-negative;

Figure 3B: purified human fibrinogen deiminated with a PAD;

- 311 = antifibrinogen monoclonal antibody 311;
- C1 = sheep antibody directed against mouse IgGs;
- C2 = sheep antibody directed against protein A;
- control sera = sera derived from patients suffering from forms of inflammatory rheumatism other than RA, or from healthy donors;
- RA sera = rheumatoid sera;
    * AFA+ = AFA-positive;
    * AFA- = AFA-negative;

Nondeiminated fibrinogen

After separation by SDS-PAGE, under the conditions described in example 1 above, the nondeiminated

fibrinogen is composed of 3 polypeptides having respective apparent molecular weights 48 kDa, 58 kDa and 69 kDa, corresponding to the expected apparent molecular masses of the α-, β- and γ-polypeptide chains making up the protein (results not given). The "311" antifibrinogen monoclonal antibody strongly recognizes the α- and β-polypeptide chains and very weakly the γ-polypeptide chain (Figure 3A).

Antisera specific for each of the α-, β- and γ-chains of fibrinogen also showed reactivity with respect to the chain against which they were respectively directed (results not shown).

Deimination of the fibrinogen

A peptidyl arginine deiminase (PAD) purified from rabbit skeletal muscle (Sigma, St. Louis, MO) was used. The human fibrinogen (Calbiochem, San Diego, CA) was incubated at the concentration of 0.86 mg/ml, in the presence or absence of PAD (7 U/mg of protein) for 2 h at 50°C, in 0.1 M Tris-HCl buffer, pH 7.4, containing 10 mM of $CaCl_2$ and 5 mM of DTT. These conditions are those which previously made it possible to generate the epitopes on a human recombinant filaggrin, recognized by AFAs [GIRBAL-NEUHAUSER et al., J. Immunol., 162, 585-594 (1999)]. The deimination was then stopped by adding 2% of SDS and heating at 100°C for 3 min.

After deimination for 2 hours, the electrophoretic mobility by SDS-PAGE of the two α- and β-polypeptides became modified and that of the γ-polypeptide remained unchanged. Specifically, the protein corresponding to the α-chain then appeared in the form of a diffuse band of 82 to 95 kDa and was immunodetected by both the "311" antifibrinogen monoclonal antibody (figure 3B) and the antiserum directed against the α-chain of fibrinogen (results not shown).

The protein corresponding to the β-chain appeared in the form of a well-defined doublet with the molecular weight of 458 kD for the lower band and 60 kD for the upper band, which was not recognized by the "311" antifibrinogen monoclonal antibody (figure 3B) but was immunodetected by the rabbit antiserum directed against the recombinant β-chain of human fibrinogen (results not shown).

No reactivity for the α-chain or for the β-chain is observed with the C1 and C2 antibodies.

Reactivity of the sera

The reactivity of the sera with respect to the α- and β-chains of nondeiminated fibrinogen proved to be zero or very weak and concerned only a few sera rarely occurring, belonging to no particular subgroup.

On the other hand, after deimination, the polypeptides corresponding to the deiminated α- and β-chains react strongly with the purified AFAs (results not shown) and with all of the 37 AFA-positive rheumatoid sera (with the exception of that which has the lowest AFA titer). Moreover, 6 AFA-negative rheumatoid sera out of 10 also clearly recognized the deiminated α- or β-polypeptides: 2 immunodetected the α-polypeptide and the β-polypeptide doublet, 3 others only detected the β-polypeptide doublet, and only 1 immunodetected exclusively the α-polypeptide. On the other hand, with the exception of a serum derived from a patient suffering from Sjögren's syndrome, which was reactive on the β-polypeptide doublet, none of the control sera immunodetected the deiminated fibrinogen.

The affinity of the AFA-positive rheumatoid sera with respect to the two deiminated α- and β-polypeptides

proved to be slightly variable from one serum to the other. Thus, 6 sera, while strongly detecting the β-polypeptide, only very weakly recognized the α-polypeptide. Similarly, 3 sera, highly reactive with respect to the α-polypeptide, did not detect the deiminated β-polypeptide. Moreover, the intensity of labeling of the two polypeptides appears, overall, to be proportional to the AFA titer of the sera. It should be noted that the sera reactive on the deiminated α and β-polypeptides of fibrinogen were also reactive with respect to high molecular weight (greater than 200 kD) polypeptides generated during the deimination of the fibrinogen. These polypeptides which clearly react with the antifibrinogen antibodies are very probably fibrinogen chain aggregates.

In conclusion, recognition of the α- and β-polypeptides of fibrinogen by rheumatoid sera is not only entirely dependent on their deimination, since the nondeiminated polypeptides are never recognized, but it is also clearly linked to the antifilaggrin reactivity of these sera. It should be noted that these deiminated polypeptides make it possible to detect with great sensitivity the AFAs present in rheumatoid sera.

These results clearly demonstrate that the antigenic targets of the ASAs in rheumatoid synovial joints are deiminated forms of the α-chain and of the β-chain of human fibrin.

CLAIMS

1. A citrullinated polypeptide derived from all or part of the sequence of the α-chain or of the β-chain of a vertebrate fibrin, by substitution of at least one arginine residue with a citrulline residue.

2. The citrullinated polypeptide as claimed in claim 1, derived from a sequence of at least 5 consecutive amino acids of the α-chain or of the β-chain of a vertebrate fibrin.

3. The citrullinated polypeptide as claimed in either of claims 1 and 2, characterized in that said vertebrate fibrin is a mammalian fibrin, preferably a human fibrin.

4. The use of a polypeptide as claimed in any one of claims 1 to 3, for diagnosing rheumatoid arthritis, *in vitro*.

5. An antigenic composition for diagnosing the presence of rheumatoid arthritis-specific autoantibodies in a biological sample, characterized in that it contains at least one citrullinated polypeptide as claimed in any one of claims 1 to 3, optionally labeled with and/or conjugated to a carrier molecule.

6. A method for detecting rheumatoid arthritis-specific autoantibodies in a biological sample, which method is characterized in that it comprises:
- bringing said biological sample into contact with at least one polypeptide as claimed in any one of claims 1 to 3, under conditions which allow

the formation of an antigen/antibody complex with the rheumatoid arthritis-specific autoantibodies possibly present;

- detecting, by any suitable means, the antigen/antibody complex possibly formed.

7. A kit for detecting rheumatoid arthritis-specific autoantibodies in a biological sample, characterized in that it comprises at least one polypeptide as claimed in any one of claims 1 to 3, and also buffers and reagents suitable for constituting a reaction medium which allows the formation of an antigen/antibody complex, and/or means for detecting said antigen/antibody complex.

8. The use of a citrullinated polypeptide as claimed in any one of claims 1 to 3, for producing a medicinal product.

9. The use as claimed in claim 8, characterized in that said medicinal product is intended to neutralize the autoimmune response associated with RA.

10. A pharmaceutical composition, characterized in that it contains, as active principle, at least one citrullinated polypeptide as claimed in any one of claims 1 to 3.

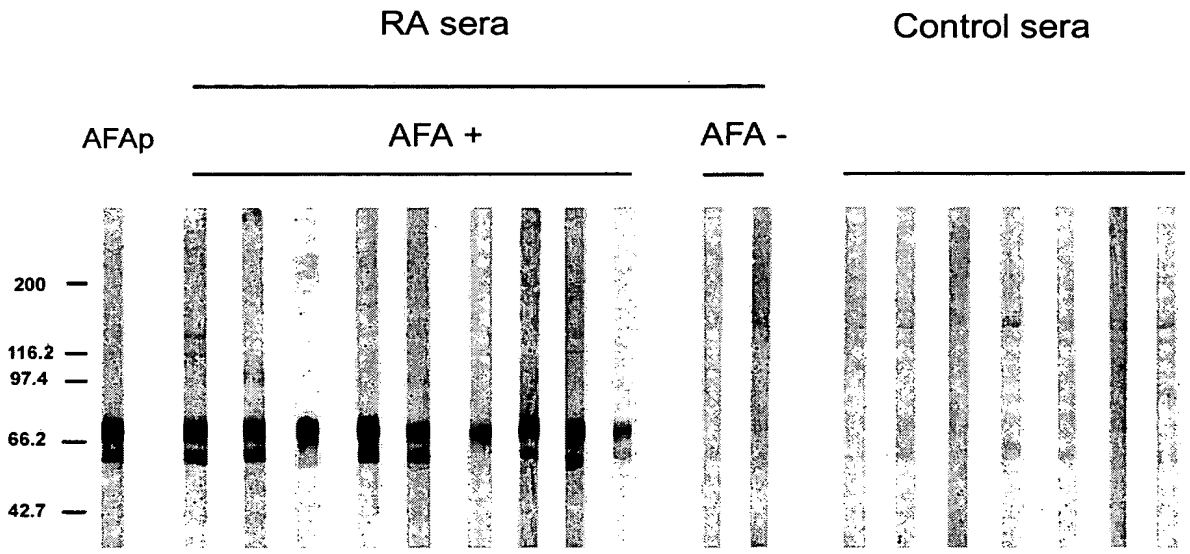


Figure 1

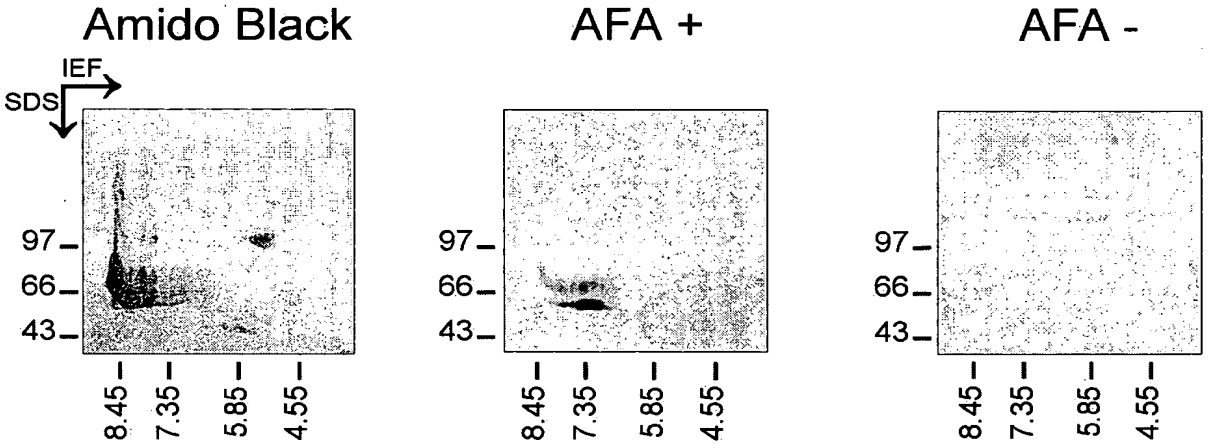
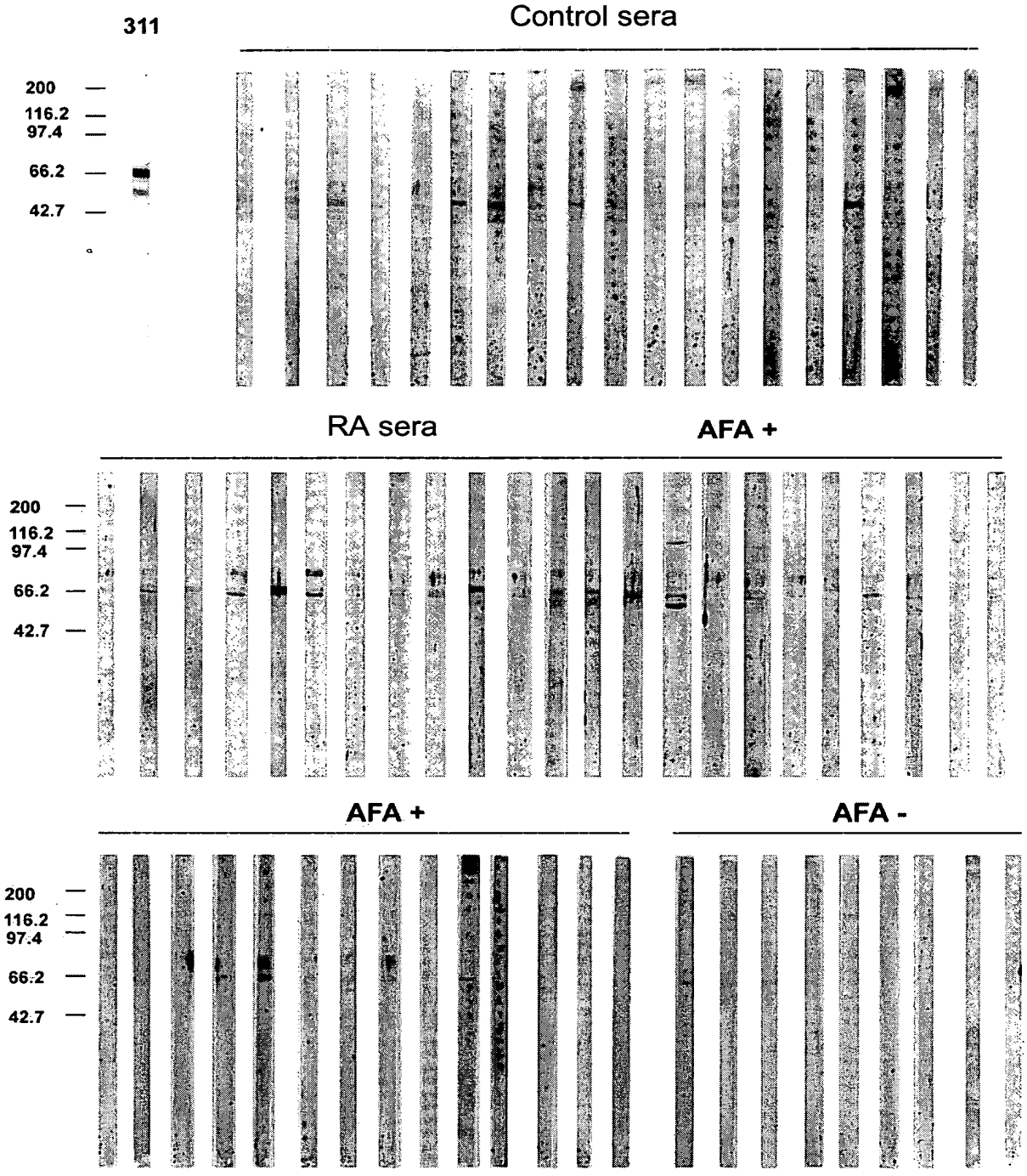


Figure 2


311
Control sera
200
116.2
97.4
66.2
42.7
RA sera
AFA +
200
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AFA +
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200
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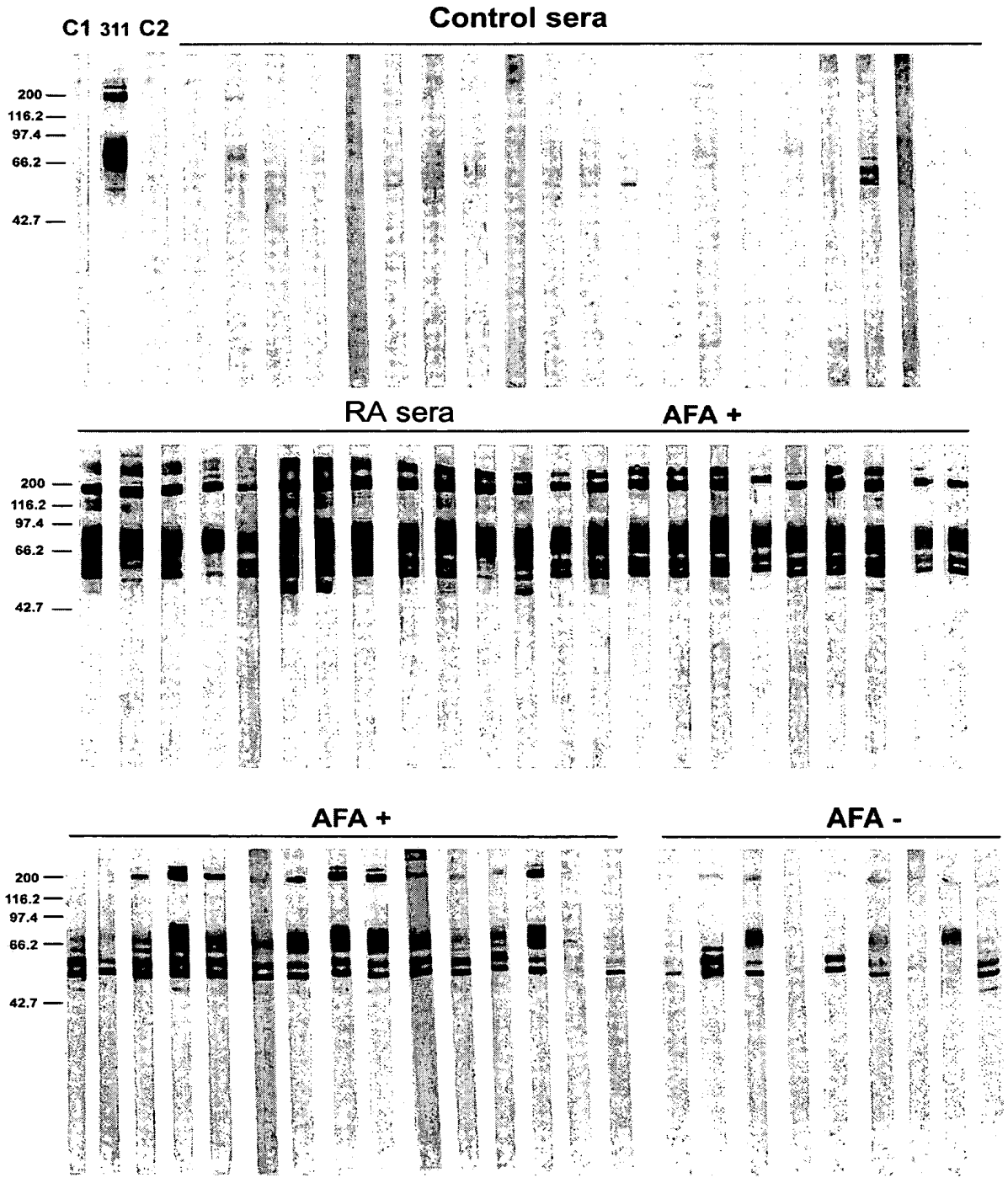
Figure 3A


C1 311 C2
Control sera
200
116.2
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RA sera
AFA +
200
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200
116.2
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Figure 3B